MEASURING OSMOSIS AND HAEMOLYSIS OF RED BLOOD CELLS

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ABBREVIATED TITLE

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ABSTRACT

Since the discovery of the composition and structure of the mammalian cell membrane, biologists have had a clearer understanding of how substances enter and exit the cell's interior. The selectively-permeable nature of the cell membrane allows the movement of some solutes and prevents the movement of others. This has important consequences for cell volume and the integrity of the cell and, as a result, is of utmost clinical importance, for example in the administration of isotonic intravenous infusions. The concepts of osmolarity

and tonicity are often confused by students as impermeant isosmotic solutes such as NaCl are also isotonic; however, isosmotic solutes such as urea are actually hypotonic due to the permeant nature of the membrane. By placing red blood cells in solutions of differing osmolarities and tonicities, this experiment demonstrates the effects of osmosis and the resultant changes in cell volume. Using haemoglobin standard solutions, where known concentrations of haemoglobin are produced, the proportion of haemolysis and the effect of this on resultant haematocrit can be estimated. No change in cell volume occurs in isotonic NaCl, and by placing blood cells in hypotonic NaCl incomplete haemolysis occurs. By changing the bathing solution to either distilled water or isosmotic urea, complete haemolysis occurs due to their hypotonic effects. With the use of animal blood in this practical, students gain useful experience in handling tissue fluids and calculating dilutions and can appreciate the science behind clinical scenarios.

INTRODUCTION

Objectives and Overview

The movement of water and small molecules across the selectively-permeable membranes of mammalian cells is a fundamental concept of physiology. These processes can be difficult for students to visualise and appreciate and it is often left to images in textbooks or online animations to explain such movements. This practical uses animal blood bathed in solutions with differing osmolarities and tonicities to explore the concept of water movement by osmosis and the resultant haemolysis that can occur when red blood cells are exposed to hypotonic solutions. Students are given the opportunity to handle body fluids, practise preparing dilutions and make accurate observations.

Background

In 1925, Gorter and Grendel (6) were the first to report the bilayer nature of the cell membrane. The structure of the cell membrane was further advanced by the work of Singer and Nicolson (18) who described the presence and location of proteins in the bilayer and developed the fluid mosaic model. In the mammalian cell membrane, the phospholipid bilayer alone is permeable to some substances such as oxygen, a small non-polar molecule, and partially permeable to water but some substances such as charged ions and glucose are

impermeant without the additional presence of protein channels and transporters in the membrane. The combined properties of the phospholipid and proteins has resulted in the use of the term the 'selectively-permeable' membrane (3,9). The extent to which solutes can cross the cell membrane dictates the tonicity of extracellular fluids and therefore the size and shape of cells from the resultant osmotic water movement (19). Knowledge of the structure and function of cell membranes and the movement of substances across the membrane is fundamental to all biomedical science disciplines and is often taught in early parts of undergraduate courses.

Osmosis is the movement of water down its osmotic gradient across a selectively-permeable membrane (5). The establishment of an osmotic pressure gradient, i.e. the pressure required to prevent the movement of water down its gradient, is a result of the difference in numbers of impermeant particles in solution on either side of the membrane (14). Water can move directly through the cell membrane; however, due to the lipid bilayer nature of the membrane this process is relatively slow. It was the discovery of water carrying pore-forming proteins known as aquaporins (16) that helped improve knowledge of how water moves from intracellular to extracellular fluid and vice versa. Water balance is crucial in homeostasis; hormones such as antidiuretic hormone (ADH) and atrial natriuretic peptide (ANP) are released in response to changes in plasma composition and volume respectively, and act on the kidney to regulate plasma osmolarity and volume.

The osmolarity of a solution is determined by the total number of particles present, known as osmolyte particles, and is not affected by the identity of these molecules (19). The higher the osmolarity of a solution, the greater the concentration of osmolytes and the physical properties of a solution such as osmotic pressure and freezing point will be dependent on the concentration of osmolytes in solution. Osmolarity is calculated from the sum of the molar concentration of each solute multiplied by the osmotic coefficient for that solute. The osmotic coefficient is determined by the degree to which a solute (e.g. an ionic compound) dissociates in solution therefore an osmotic coefficient of '1' indicates that the solute completely dissociates in solution.

For example, to calculate the osmolarity of a 0.9% weight/volume NaCl (MW 58.44) solution firstly the molarity is calculated by:

Molarity of a % w/v solution (M) = % solution in g/litre ÷ molecular mass of the solute

0.154 M = 9 g/litre ÷ 58.44 g/mol

To calculate the osmolarity, given that NaCl dissociates into two ions (Na^+ and Cl^-) in solution and has an osmotic coefficient of 0.93, the following equation is used:

Osmolarity of solution (osmol/l) = molarity (M) x number of osmoles produced by dissociation x osmotic coefficient

0.286 osmol/l or 286 mosmol/l = 0.154 M x 2 x 0.93

Osmolarity and tonicity are often used interchangeably by students but they are not the same. Tonicity refers to the effect a solution has on cell volume as a result of the permeability of the membrane to that solute. Tonicity is therefore determined by the osmolarity and whether the solute can cross the cell membrane; it is the concentration of the impermeant solutes alone that determines tonicity. When comparing fluid concentrations to that of extracellular body fluid, the terms isotonic, hypertonic and hypotonic are used rather than osmolarity as they describe the effect the solution has on cell volume which is of physiological significance. The tonicity will result in: no net movement of water (isotonic), net flow of water out of a cell (hypertonic), or net flow of water into a cell (hypotonic). Two solutions that are isosmotic may not be isotonic. A key example is isosomotic urea and isosmotic NaCl. Both urea and NaCl have the same osmolarity, having the same total number of osmolyte particles, however the membrane is permeable to urea which will freely diffuse across the cell membrane and impermeable to NaCl. An isosmotic urea is therefore hypotonic compared to an isosmotic and isotonic solution of the impermeant NaCl. As a result, the volume of a cell is determined by the solution in which it is being bathed **and** whether the cell's membrane is permeable to the solute. If a membrane is not equally permeable to all solutes then a difference in water movement will be observed that is not explained by osmolarity alone and hence an additional term, tonicity, is required.

Hypotonic solutions lead to cell swelling and eventual rupture or lysis if the resultant osmotic movement of water is great enough. In the case of red blood cells this is referred to as haemolysis (4).

Knowledge of osmosis and tonicity is crucial in understanding the movement of fluids in the body. These concepts are fundamental in normal physiological processes; one example is that of water reabsorption in the kidney as increases in osmolarity are detected by the hypothalamus and stimulate the secretion of ADH resulting in greater water retention and excretion of more concentrated urine (7). Osmosis and tonicity are important clinically as the failure of the body to respond to changes in osmolarity, or the failure to release ADH, result in the condition diabetes insipidus. Another important concept is the diagnosis of the different types of dehydration and the administration of appropriate intravenous fluids (2). In this practical, using easy to obtain red blood cells as model cells (1), students can explore the concepts of membrane permeability, osmosis, osmotic pressure, tonicity and haemolysis whilst also learning key laboratory skills such as making dilution series and handling tissue fluids.

Learning Objectives

After completing this activity, the student will be able to:

1. CONTENT KNOWLEDGE: Define key terms used in explaining concentration, osmolarity, osmotic pressure and tonicity
2. CONTENT KNOWLEDGE: Calculate the osmolarity of a solution
3. CONTENT KNOWLEDGE: Describe and explain the consequences of bathing red blood cells in solutions of differing tonicity
4. PROCESS SKILLS: Handle mammalian blood samples safely
5. PROCESS SKILLS: Prepare standard saline solutions
6. PROCESS SKILLS: Measure haematocrit and estimate haemoglobin concentration
7. PROCESS SKILLS: Carry out experiments with careful planning, accurate observation and recording of results

Activity Level

This activity is used to teach students in their first year of undergraduate study in physiology. This practical is used on our Physiological Sciences programme and Veterinary Science programme but would also be suitable for other biomedical science or healthcare professional programmes such as medicine.

Prerequisite Student Knowledge or Skills

Before undertaking this activity, students should have a basic understanding of:

Homeostasis and the proportions of fluid in intracellular and extracellular compartments

The definition of a solute, a solvent and a solution

The concept of osmosis and the movement of water across a selectively-permeable membrane

Students should know how to:

Perform basic calculations to work out volumes required for concentrations

Use pipettes to create serial dilutions from stock solutions

Collect data carefully and accurately

Observe safe laboratory practices

Time Required

This practical is run in a 3 hour laboratory time-slot. The practical is completed within one session; however, it is expected that students complete their pre-reading of the laboratory notes which explain the concepts of osmolarity, tonicity and how to calculate osmolarity (to aid in achieving content learning objectives 1 and 2) and an online pre-practical quiz before they come to the practical. This preparation work is expected to take around 1 hour.

METHOD

Equipment and Supplies

The following equipment and supplies are needed:

Solutions

1. Distilled water (20 ml per pair of students)
2. 2.7% w/v NaCl solution (2.7 g NaCl per 100 ml of distilled water) (20 ml per pair of students plus that required for non-haemolysed blood preparation). This stock solution is used to prepare all other NaCl solutions in the experiment
3. Isosmotic urea solution (17.1 g/l) (5 ml per pair of students plus that required for haemolysed blood preparation)
4. Fresh mammalian blood. This blood is referred to for the rest of the experiment as non-haemolysed blood. We find that there are no appreciable differences in the outcome of the experiment depending on which species blood is used although values of haemolysis can vary. Obtaining mammalian blood supplies can be problematic if obtained locally direct from an abattoir; however, blood can also be purchased online for example http://www.rockland-inc.com/blood-products.aspx. For a class of around 200 students working in pairs, approximately 1.5 L of blood is required (approximately 11 ml blood per pair of students and allowing extra for repeat experiments if required). The blood must be heparinised before use to prevent clotting by the addition of heparin sodium (5,000 I.U/ml per 1.5 litres blood). This blood is then used to produce the haemolysed and non-haemolysed blood as follows:
5. Haemolysed blood. To prepare the haemolysed blood in manageable volumes, 250ml of non-haemolysed blood is measured into a 600ml beaker together with 250 ml urea solution (17.1g/l) and stirred. The tonicity of the urea and resultant osmotic water movement results in haemolysis of the cells and this will form the blood used for the production of the haemoglobin standards that will be used to assess the degree of haemolysis in the experiment. Decant 10 ml of the haemolysed blood into 50 centrifuge tubes (one per pair of students), labelled H for haemolysed blood and centrifuge at 6000 rpm for 2 minutes. Repeat depending on quantities of blood required i.e. if 1l required repeat once.
6. Non-haemolysed blood. To prepare the non-haemolysed blood in manageable volumes, 275 ml of non-haemolysed blood (from the original heparinised fresh mammalian blood) is prepared by the addition of 275 ml of 0.9% w/v saline and stirred gently. This forms the non-haemolysed blood which will be used

for the main part of the experiment at an equal concentration to the haemolysed blood. Decant 11 ml of the non-haemolysed blood into 50 centrifuge tubes (one per pair of students) labelled N for non-haemolysed blood. Repeat depending on quantities of blood required i.e. if 1l required repeat once.

An assumption is made that the haemoglobin concentration of the original blood sample is 15 g/dl; but as the haemolysed blood is diluted 1:1 with isosmotic urea (17.1g/l) and the equivalent non-haemolysed blood is diluted 1:1 with isosmotic (0.9% w/v NaCl), the haemoglobin concentration of both blood samples is therefore assumed to be 7.5 g/dl (75 g/litre).

Equipment

1. 600 ml glass beakers (2 for blood preparation)
2. 500 ml measuring cylinders (2 for blood preparation)
3. Stirring rods (2 for blood preparation)
4. 25 ml glass beakers for water, 2.7% w/v NaCl and urea distribution (3 per pair of students)
5. 1.5 ml plastic Eppendorf tubes with hinged cap (11 per pair of students)
6. 10 ml plastic centrifuge tubes with cap (10 per pair of students)
7. Centrifuge tube racks (1 per pair of students)
8. 75 µl glass microhaematocrit tubes (Hawksley catalogue no. 01603) (6 per pair of students)
9. Plasticine
10. Centrifuge with centrifuge tube rotor and microhaematocrit tube rotor (Hettich EBA21 centrifuge with 1416 rotor and 1450 haematocrit rotor)
11. Haematocrit readers (Hawksley) or 30 cm rulers (a number of readers/rulers can be shared between pairs of students)
12. 1.5 ml disposable plastic pipettes or equivalent Gilson pipettes if available (3 disposable pipettes per pair of students)
13. Marker pens (1 per pair of students)
14. White paper (1 sheet per pair of students)

Human or Animal Subjects

The animal blood used in this experiment is obtained as a by-product from a local abattoir and therefore the animals are not slaughtered for the purpose of this experiment.

Instructions

Preparation prior to the practical

In advance of the class, students must calculate (1) the volume of distilled water and 2.7% w/v NaCl stock solution required to produce 9 ml each of 0.9 and 0.45% w/v saline solutions; (2) the volumes of haemolysed blood and 0.9% w/v NaCl (ml) required to produce 1.5 ml % haemoglobin concentrations and (3) the haemoglobin concentration (g/dl) in results tables 1-3 provided in their lab books.

In our programmes this is the first practical that students will have had to calculate and make serial dilutions and handle blood; two key, but challenging, transferable skills.

Making saline solutions and haemoglobin standards

We recommend that students carry out this practical working in groups of two or three. Students begin the practical by making a set of standard solutions of haemolysed blood of known haemoglobin concentration to use later in the experiment which they will compare against the unknown haemoglobin containing solutions they will produce. Haemolysed blood is used to create these haemoglobin standards as this contains red blood cells that have already fully lysed in urea and all the haemoglobin has been released into the solution. The steps below take the students through the practical:

1. Using the 2.7% w/v NaCl and 1.5 ml pipettes provided, prepare 9 ml each of 0.9% w/v NaCl and 0.45% w/v NaCl solutions in two labelled 10ml plastic centrifuge tubes from the dilutions calculated below in Table 1 (values underlined are calculated by the students in advance of the class).

Table 1: Dilutions calculations for saline solutions

2. Using the marker pen, label five Eppendorf tubes – 100%, 66%, 33%, 7% and 1% which will represent the percentage of haemolysed blood to be added to these Eppendorf tubes. Using the volumes calculated in Table 2, use 1.5 ml pipettes to add the appropriate volumes of 0.9% w/v NaCl solution and haemolysed blood to each labelled Eppendorf tube (values underlined are calculated by the students in advance of the class). Gently invert the tube containing the haemolysed blood before use to ensure the blood is evenly mixed and once filled also invert each Eppendorf tube to ensure mixing.

Table 2: Dilutions calculations for haemoglobin standards from haemolysed blood

The calculations performed in Table 3 provide reference haemoglobin concentrations for each haemoglobin standard (values underlined are calculated by the students in advance of the class).

Table 3: Haemoglobin concentration in each standard solution

3. Lay out the five mixed haemolysed blood/0.9% w/v NaCl solutions in the Eppendorf tubes on a blank sheet of white paper to observe the colours. The colours of the haemoglobin standards should range from translucent pink to translucent red and should look similar to Figure 1. These haemoglobin standards will be used later in the experiment and should be kept to one side until then.

Figure 1: The haemoglobin standards produced from haemolysed blood with 1% haemolysed blood to the left of the image and 100% haemolysed blood on the right. Image courtesy of the University of Bristol.

Investigating the effects of tonicity on red blood cells

The next part of the experiment investigates the effects of membrane-permeable and membrane-impermeable solutions of differing concentrations on whole red blood cells using the non-haemolysed blood sample. Both haematocrit and % haemolysis will be estimated. The haematocrit will indicate the degree to which red blood cells swell or shrink when exposed to the different solutions but does not take into account

if haemolysis has occurred. Percentage haemolysis gives a measure of the degree of haemolysis of the samples and can be used to determine if red blood cells have swollen and burst. Haematocrit alone cannot distinguish between cell shrinkage and a combination of swelling and lysis.

4. Label an additional six 10 ml plastic centrifuge tubes from 1-6. Gently invert the tube containing the non-haemolysed blood several times before use to ensure an even suspension of red blood cells.

5. Prepare the centrifuge tubes as follows:

Tube 1 – 1.5 ml non-haemolysed blood + 1.5 ml 2.7% w/v NaCl

Tube 2 – 1.5 ml non-haemolysed blood + 1.5 ml 0.9% w/v NaCl

Tube 3 – 1.5 ml non-haemolysed blood + 1.5 ml 0.45% w/v NaCl

Tube 4 – 1.5 ml non-haemolysed blood + 1.5 ml distilled water

Tube 5 – 1.5 ml non-haemolysed blood + 1.5 ml isosmotic urea

Tube 6 – 3 ml non-haemolysed blood

When filled, gently invert the centrifuge tubes several times to ensure the blood is mixed and leave for 10 minutes before proceeding with the next step.

6. The blood solutions are then prepared for centrifuging to allow the measurement of the packed cell volume (haematocrit) of each sample. Label six glass microhaematocrit tubes 1-6 to correspond to the samples in the plastic centrifuge tubes. In turn, invert each centrifuge tube several times to ensure even dispersal of red blood cells and then dip the corresponding microhaematocrit tube in the blood until capillary action has filled the glass tube. Seal the bottom of the microhaematocrit tube with a small plug of plasticine by twisting the bottom of the tube in a tray of plasticine.

7. Centrifuge the microhaematocrit tubes at 6,000 rpm for 2 minutes using the microhaematocrit tube rotor until the cells have packed together at the bottom of the tube leaving the fluid (supernatant) above.

It is not expected that the centrifuges are operated by the students, in our laboratory students bring their samples to the shared laboratory centrifuges and these are run by experienced demonstrators or technicians.

Measuring haematocrit

8. After centrifuging, measure the haematocrit of each sample using a haematocrit reader and read off the % haematocrit. If haematocrit readers are difficult to obtain, a ruler can be used instead. By this method, measure the total length of the column of fluid and the length of the column of packed cells and calculate the proportion of the total column that is made up of packed cells at the bottom. This percentage is the haematocrit. Record the haematocrit readings in the *observed haematocrit* column in Table 4.

Table 4: Haematocrit measurements for non-haemolysed blood

9. With the exception of tube 6, the haematocrit readings measured are for blood diluted 50:50 with a saline solution. Therefore complete the *dilution factor* column with a dilution factor of 2 for tubes 1-5 and a dilution factor of 1 for tube 6. To calculate the true haematocrit values, complete the final *corrected haematocrit for non-haemolysed blood* column in Table 4 by using the following equation:

Corrected haematocrit = observed haematocrit x dilution factor

Estimating haemolysis

10. Following the measurement of haematocrit, estimate the percentage of haemolysis of the red blood cells in the various solutions. To do this, centrifuge the remaining contents of the 6 plastic centrifuge tubes at 6000 rpm for 2 minutes using the centrifuge tube rotor. Take six clean 1.5 ml plastic Eppendorf tubes also labelled 1-6, pipette 1.5 ml of the supernatant from each correspondingly labelled centrifuge tube into the labelled Eppendorf tube taking care not to disturb the red blood cell pellet at the bottom of the tube.

11. The colours of the six samples of supernatant can then be compared to that of the known haemoglobin standard solutions prepared at the beginning of the practical. Using the colours of the known haemoglobin standard solutions as a scale, estimate the concentration of observed supernatant haemoglobin with the darker the colour of the sample indicating the greater the amount liberated haemoglobin in the supernatant and hence the greater degree of haemolysis. Using the known haemoglobin concentrations (g/dl) calculated

in Table 3, record these observations for tubes 1-6 in the *observed supernatant [Hb]* column of Table 5. If the colours are not exact matches, estimate whereabouts between the two standards the concentration falls.

12. To convert the observed haemoglobin concentration into an estimated percentage of haemolysis of the red blood cells, with the exception of the non-haemolysed blood sample which contained 7.5 g/dl haemoglobin, the blood in the other mixtures was diluted 50:50 and therefore contained half the original haemoglobin. To estimate the amount of haemolysis that occurred in each sample use the following calculations and complete the *estimated % haemolysis* column of Table 5:

Diluted samples estimated % haemolysis = [observed supernatant Hb concentration / 3.75] x 100

Non-haemolysed blood estimated % haemolysis = [observed supernatant Hb concentration / 7.5] x 100

13. The corrected haematocrit recorded in Table 4 was generated by non-haemolysed red blood cells only as these were the whole cells that would have made up the packed cell volume in the haematocrit tubes. To correct for haemolysis in each sample and allow an estimate of what haematocrit would be had there had been no cell lysis, use the following calculation and complete the final column (*corrected haematocrit*) of Table 5:

Corrected haematocrit (%) = [100 / (100 – estimated % haemolysis)] x corrected haematocrit for non-haemolysed blood (%)

Table 5: Estimated haemolysis and final corrected haematocrit

14. When all data have been collected, each group should pool their final corrected haematocrit (%) data from Table 5 with the rest of the class using a spreadsheet on a central computer to ensure that group data can be distributed for more comprehensive analysis following the class.

Troubleshooting

A common student mistake in this practical is the incorrect or lack of labelling of tubes and pipettes containing the different solutions during the various steps undertaken. As a result, students lose track of the contents of tubes they are testing and find their results are meaningless. This is an important error to impress upon the students as, if this is kind of mistake occurs in a clinical setting, the outcome could be life-threatening. Trained demonstrators should be on hand to spot mistakes early and help students rectify them as soon as possible.

Students also often find it difficult to perform the correct calculations to work out dilutions (15). It is recommended that students are encouraged to attempt these calculations (Tables 1-3) prior to the practical and come prepared to have these calculations checked by a demonstrator in the practical before proceeding.

Safety Considerations

Despite the risk to humans from animal blood being extremely low, when dealing with blood, standard safety precautions must be taken to minimise the risk of infection. At all times in the laboratory general laboratory safety rules must be followed including wearing a laboratory coat and using disposable gloves and hand washing before leaving the laboratory. Any spilled blood or fluids must be wiped up immediately and disposed of in waste bags provided. All sharps should be disposed of in a sharps box.

Unless the students are already trained and experienced in using centrifuges, the centrifuge should only be operated by trained personnel and students should not be left to spin their samples unsupervised. The centrifuge should be inspected for damage regularly. When using the centrifuge, ensure the tubes are undamaged, firmly sealed and haven't been overfilled. When placing the tubes in the rotor they must be balanced and the lid must never be opened while the rotor is moving. The centrifuge should not be left unattended during use.

RESULTS

Expected Results

Non-haemolysed blood

Tube 6 which contains the non-haemolysed blood sample prepared in step 5 should be used as a control and reference point against which to compare any changes to haematocrit in the other blood samples (tubes 1-5) that were exposed to permeant and non-permeant solutes. Completed sample data tables (Tables 6 and 7) are given here from experiments carried out using pig blood but caution should be taken when making direct comparisons to the values obtained as, although the relative changes should be the same, the actual values can vary greatly depending on the blood sample used.

The effects of hypertonic NaCl

In step 5, non-haemolysed blood was exposed to 2.7% w/v NaCl solution which has an osmolarity of 859 mosmol/l and is hypertonic relative to plasma (tube 1). When red blood cells are placed in a hypertonic solution, the higher effective osmotic pressure of the bathing solution compared to the intracellular fluid results in water moving down its osmotic gradient and a net movement of water out of the cell via osmosis (10). The red blood cells therefore lose their normal biconcave shape and shrink or crenate. This collapse leads to a decrease in the packed cell volume, or haematocrit, of the solution in comparison to that of the non-haemolysed blood as the cells take up less space due to the rapid loss of water. Very little haemolysis of the red blood cells in the solution should be observed as no cells have taken on an additional water load and burst or haemolysed; however, a few cells may have been damaged during handling and release some haemoglobin.

The effects of isotonic NaCl

In step 5, non-haemolysed blood was exposed to an isotonic solution of 0.9% w/v NaCl (osmolarity 286 mosmol/l) (tube 2). This environment has an even distribution of osmolyte particles across both sides of the cell membrane as intracellular fluid also has an osmolarity around 286 mosmol/l. There is therefore no net water movement between the bathed red blood cells and the NaCl solution. The haematocrit of the solution

should be unaffected and the value similar to that of the non-haemolysed blood. Similarly, little if any haemolysis of the red blood cells should have occurred.

The effects of hypotonic NaCl

In step 5, non-haemolysed blood was exposed to a low osmolarity (143 mosmol/l) hypotonic solution (0.45% w/v NaCl) (tube 3). When red blood cells are exposed to these conditions where there is a higher concentration of water and lower effective osmotic pressure outside the cell compared to the intracellular fluid, this results in net movement of water into the cells via osmosis (11). The cells will increase in size and some may haemolyse. In this sample therefore a small proportion of haemolysis should have been observed with increased haemoglobin in the supernatant when compared to the whole blood and the remaining cells which hadn't lysed would increase in size causing the haematocrit to increase.

The effects of distilled water

In step 5, the cells in tube 4 that were bathed in distilled water underwent complete haemolysis and the estimated % haemolysis should have been 100%. With no ions present in the bathing solution this solution was very hypotonic resulting in net movement of water into the red blood cells via osmosis causing all the cells to lose the integrity of their membranes and to haemolyse releasing haemoglobin into the supernatant, hence the strong red colour of the sample. The resultant corrected haematocrit was 0% as there were no remaining complete red blood cells to contribute to pack cell volume. Comparing the results of distilled water (tube 4) and 0.45% w/v (tube 3) is a clear example of how the osmotic fragility or susceptibility of red blood cells to haemolysis depends on the degree of hypotonicity of the bathing solution.

The effects of isosmotic urea

In contrast to NaCl, the membrane is permeable to urea. In Step 5 when red blood cells were bathed in isosmotic urea (286 mosmol/l) (tube 5), the effects of the permeability of the membrane to urea on both haematocrit and degree of haemolysis were very different than when red blood cells are exposed to isosmotic NaCl (tube 2). In the presence of an isosmotic urea solution, the red blood cells underwent

complete haemolysis with a corrected haematocrit of 0%. This is because although isosmotic, the urea solution is not isotonic as urea can freely diffuse across the cell membrane into the cell via passive diffusion and through urea transporters (20, 21). This leads to a change in cell volume as a result of osmotic water movement (13). The isosmotic urea solution is therefore hypotonic because the reflection coefficient of the membrane (permeability) for urea is 0.024 compared to a reflection coefficient of the membrane of 0.3 for NaCl. If the membrane is completely impermeable to a solute the reflection coefficient would be 1. The consequence of this is that the effective osmotic pressure of a urea solution is lower than that of NaCl of the same osmolarity and, as a result, the osmotic gradient across the cell membrane is increased and water moves into the red blood cells via osmosis causing the cell membrane to rupture and the cell to haemolyse. Conversely, NaCl dissociates into Na^+ and Cl^- particles that cannot cross the cell membrane and therefore generate an equal effective osmotic pressure between the extracellular fluid and the intracellular fluid. Under these conditions the osmotic gradient across the cell membrane is maintained and the solution is both isosmotic and isotonic. The same strong red colour of the urea sample in tube 5 should have been observed as that of the distilled water sample in tube 4 as there is 100% haemolysis and 0% corrected haematocrit.

Conclusions

The observations and conclusions that should have been drawn from this practical are fundamental to understanding basic cell physiology. A good grasp of the concepts covered by this practical will help students appreciate the fact that cell membranes are indeed selectively-permeable and that the tonicity and osmolarity of fluids affect cell size and structure. This is essential in understanding the concept of homeostasis and will be referred to in later parts of many physiology courses including during study of the gastrointestinal tract, regulation of NaCl by the nephron in the renal system and in particular the effect of dehydration on the whole body.

Caution must be taken with the practical to ensure students observe the expected results, common mistakes such as poor labelling of samples and contamination with urea due to pipette confusion can lead to students

obtaining results that may not be as anticipated. Careful supervision of students and pooling of data to analyse the class averages should help prevent this.

Table 6: Completed sample results of haematocrit measurements for non-haemolysed blood

Table 7: Completed sample results of estimated haemolysis and final corrected haematocrit

Figure 2: Mean corrected haematocrit values of blood following exposure to permeable and non-permeable solutions (n=64). Error bars represent SEM.

Misconceptions

From early on in many physiology-based courses, students struggle with the concepts of osmolarity and tonicity and find it difficult to relate them to the direction of water movement. This practical can help students to visualise different solutions and the effects that these can have on red blood cells. By being able to see the colour change of the non-haemolysed blood samples mixed with various solutions they can relate the theory of osmosis to what has happened to the cells when water moves out of the red blood cells in a hypertonic solution (tube 1), when there is no net movement of water in an isotonic solution (tube 2) and when water moves into the cells in a hypotonic solution (tube 3). The practical also helps students with misconceptions surrounding haemolysis and haematocrit. The students often mistake the contents of the tubes (4 and 5) containing distilled water and urea as having 100% haematocrit due to the dark colouration of the whole sample with little or no visible plasma band rather than the product of 100% haemolysis due to the effects of the tonicity. Trained demonstrators should be on hand to ensure that students are able to relate their findings to the learning outcomes and a whole class tutorial on the outcomes of the experiments and their meanings is scheduled for one week after the practical class.

Evaluation of Student Work

As discussed, there are a number of ways in which students may not get the results expected. To ensure that students have access to some representative data on which to perform any post-practical analysis, students are expected to pool their results with the rest of the group to produce group data for the class prior to the finish and this is then shared online with the students to use immediately after the class.

We assess our students on this work through the submission of an online post-practical assessment. This takes the form of multiple choice questions on the background physiology, method, results and physiological significance of the findings. Questions included in the assessment test whether students have grasped the direction of fluid movement in the presence of different solutions and the physiological reasons for this. With regards to further data analysis, students are expected to plot the class findings in graphical form. They are instructed to produce a column/bar graph of the final corrected haematocrits (%) from all solutions with the error bars as the standard error of the mean and this is uploaded as part of their online post-practical assessment (see Figure 2). Students are also given information on how to calculate osmolarity and are expected to perform calculations themselves.

Inquiry Applications

As this practical is run at the start of the students' exploration of physiology at undergraduate level there is very limited inquiry in this practical and would be considered 'Methods' level. The questions being explored and the procedure being followed are clearly set out by the teacher running the practical. Students carry out the practical, with assistance from demonstrators and analyse the data during the session.

However, there is scope for this practical to become more student-centred and to be used at a higher inquiry level by making a number of modifications to the protocol. These could include giving the students less rigid instructions on how and what haemoglobin standard solutions to produce and allowing them to make a wider range of standard solutions to allow more accuracy in estimating the final degree of haemolysis. They could also be asked to predict the impact of the different solutions on the haematocrit and haemoglobin concentration and subsequently test these predictions. More sophisticated techniques could also be used to

measure haemolysis in the supernatant such as spectrophotometry and using light microscopy to visualise the red blood cells after they have undergone crenation or haemolysis as opposed to the estimates made in this experiment by eye.

This practical could be incorporated into a number of different biomedical programmes, from Biology/Physiology/Biochemistry honours programmes to aid in the understanding of the fundamental concepts of cell transport and membrane structure as well as developing vital scientific skills including handling blood and performing serial dilutions. Professional disciplines such as medicine and veterinary science would also benefit from this practical to further explore the concepts of osmotic fragility and the administration of intravenous fluids and the clinical implications that these can have.

The concept of osmotic fragility could be explored further by using a series of hypotonic solutions and recording % haemolysis. From these data an osmotic fragility curve could be plotted to explore the internal pressures exerted on the cell membrane when water diffuses into a cell. Students could further consider how the shape of red blood cells e.g. sickle cells may affect the osmotic fragility of red blood cells (8) and how haemolytic diseases such as thalassemia and hereditary spherocytosis are a result of changes in osmotic fragility both by extending this practical and the use of further resources (12).

Further examples of the differences between tonicity and osmolarity and the effect of permeable solutes can be used, for instance with the addition of glucose, a particularly clinically relevant solute with regards to intravenous administration of fluids. The clinical application of an understanding of this concept can be emphasised including those surrounding patient safety (17).

ADDITIONAL RESOURCES

For additional information on this topic, any undergraduate level physiology textbook should provide relevant background information required to understand the theory this practical is based upon.

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